Molecules and Cells

The Peroxisomal Localization of Hsd17b4 Is Regulated by Its Interaction with Phosphatidylserine

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https://doi.org/10.14348/molcells.2021.2217
www.molcells.org

Phosphatidylserine (PS), a negatively charged phospholipid exclusively located in the inner leaflet of the plasma membrane, is involved in various cellular processes such as blood coagulation, myoblast fusion, mammalian fertilization, and clearance of apoptotic cells. Proteins that specifically interact with PS must be identified to comprehensively understand the cellular processes involving PS. However, only a limited number of proteins are known to associate with PS. To identify PS-associating proteins, we performed a pulldown assay using streptavidin-coated magnetic beads on which biotin-linked PS was immobilized. Using this approach, we identified Hsd17b4, a peroxisomal protein, as a PS-associating protein. Hsd17b4 strongly associated with PS, but not with phosphatidylcholine or sphingomyelin, and the Scp-2-like domain of Hsd17b4 was responsible for this association. The association was disrupted by PS in liposomes, but not by free PS or the components of PS. In addition, translocation of PS to the outer leaflet of the plasma membrane enriched Hsd17b4 in peroxisomes. Collectively, this study suggests an unexpected role of PS as a regulator of the subcellular localization of Hsd17b4.

Keywords: efferocytosis, exposure, Hsd17b4, interaction, peroxisome, phosphatidylserine

INTRODUCTION

One key feature of the plasma membrane is the asymmetrical distribution of lipids. Charged phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), exclusively localize to the inner leaflet of the plasma membrane, whereas neutral phospholipids, such as phosphatidylcholine (PC), mainly localize to the outer leaflet (van Meer et al., 2008). The asymmetrical distribution of charged phospholipids is maintained by the actions of scramblases and flippases. Scramblases induce exposure of charged phospholipids on the cell surface by mixing lipids between the leaflets, whereas flippases translocate them from the cell surface to the cytosolic face of the plasma membrane (Clark, 2011). Therefore, PS, which is exclusively located in the inner leaflet of the plasma membrane, can be exposed in a regulated manner, and exposure of PS on the cell surface generates a signal for various cellular responses (Kay and Grinstein, 2013).

One of the best-known cellular responses mediated by PS exposure is phagocytosis of apoptotic cells, referred to as efferocytosis. Scramblases are activated while flippases are inactive during apoptosis, leading to exposure of PS on the surface of apoptotic cells (Nagata et al., 2016; Sakuragi et al., 2019; Segawa and Nagata, 2015; Segawa et al., 2014). Apoptotic cells with exposed PS are recognized by

Received 7 December, 2020; revised 20 March, 2021; accepted 5 April, 2021; published online 27 April, 2021

eISSN: 0219-1032

PS receptors on phagocytes or by binding of secreted proteins such as Mfge8 and Gas6 to a receptor on phagocytes, which activates signaling pathways downstream of the receptors to phagocytose apoptotic cells (Moon et al., 2020b; Ravichandran and Lorenz, 2007). PS is exposed not only on dying cells, but also on living cells. Activated platelets, aged erythrocytes, and some cancer cells have exposed PS on their surfaces, which generates a signal for cellular responses (Qadri et al., 2017; Riedl et al., 2011; Rival et al., 2019; Zwaal et al., 1998). Coagulation is one example of a cellular process in which translocation of PS in living cells serves as a signal to generate a downstream response. Activated platelets release vesicles on which PS is exposed. This promotes assembly of the prothrombinase complex and facilitates activation of the complex, which is required to generate thrombin from prothrombin (Lentz, 2003).

A limited number of PS-binding proteins have been identified. One interesting characteristic of these proteins is that calcium is often crucial for their interactions with PS, although not all PS-binding proteins require calcium to interact with PS (Stace and Ktistakis, 2006). For example, calcium is essential for the PS receptor to recognize PS on apoptotic cells. Depletion of calcium abrogates binding of the PS receptor to PS. In addition, Annexin A5, which is the best-known PS-binding protein and is commonly used to label PS on apoptotic cells, also requires calcium for binding to PS (Santiago et al., 2007; Swairjo et al., 1995; Voges et al., 1994). PS-binding proteins range from cytosolic to extracellular proteins, which indicates that translocation of PS across the membrane may function as a switch to turn signals on and/or off (Hanayama et al., 2002; Lomasney et al., 1999; Miyanishi et al., 2007; Orr and Newton, 1992; Park et al., 2007).

In this study, due to the significance of PS as well as its associated proteins and the limited number of identified PS-binding proteins, we screened for PS-binding proteins using a pulldown assay with streptavidin-coated magnetic beads (SCMBs), on which biotin-linked PS was immobilized. One candidate identified by the screen was Hsd17b4, which is a peroxisomal protein involved in peroxisomal fatty acid metabolism. Hsd17b4 associated with PS through its Scp-2-like domain, and its association with PS was impaired by PS liposomes, but not PC liposomes, free PS, or the components of PS. Exposure of PS on the outer leaflet of the plasma membrane using A23187, a calcium ionophore, increased the peroxisomal localization of Hsd17b4. In addition, Hsd17b4 was enriched in peroxisomes of apoptotic cells on which PS was exposed. Taken together, these data suggest that translocation of PS regulates the subcellular localization of Hsd17b4.

MATERIALS AND METHODS

Primary mouse bone marrow derived macrophage preparation

For preparing mouse primary bone marrow-derived macrophages (BMDMs), bone marrow cells from the hind legs of C57BL/6 mice were collected and incubate with medium containing 20% L929 conditioned medium, 10% fetal bovine serum (FBS) (35-015-CV; Corning, USA) and 1% Penicillin-Streptomycin-Glutamine (PSQ) (GIB-10378-016; Gibco, Ireland) in RPMI (31800-022; Gibco) for 7 days. More than 95% of cells after differentiation were double positive for F4/80 and CD11b.

Cell culture and transfection

293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (12800082; Gibco) and LR73 cells were cultured in alpha-MEM media (50-012-PC; Cellgro, Finland). All cell culture media contains 10% FBS and 1% PSQ. For transfection of 293T and LR73 cells, calcium phosphate and Lipofectamine 2000 (11668019; Invitrogen, USA) were used, respectively.

Plasmids and antibodies

All plasmids in this study were constructed through polymerase chain reaction (PCR)-base strategy and sequenced to confirm their identity. Full cDNA sequence of mouse *Hsd17b4* was purchased from Darmacon (USA) (MMM4769-202765308). Full sequence *Hsd17b4* was constructed in the pEBB-Flag and pEBB-GFP vector (Min et al., 2020). The untagged protein expressing construct was generated as well. In order to identify PS binding region of Hsd17b4, the three domains of Hsd17b4 were constructed in the pEBG-GST vector. GST-Hsd17b4$^{1-305}$, GST-Hsd17b4$^{321-621}$, and GST-Hsd17b4$^{633-730}$ contain the hydroxyacyl-CoA dehydrogenase domain, Enoyl-CoA hydratase 2 domain, and the SCP2-like domain of Hsd17b4. HA-Tim-4^{IgV} was previously reported (Lee et al., 2019).

The antibodies used in this study were anti-Hsd17b4 (15116-1-AP [Proteintech, USA] and NBP2-46005 [Novus, USA]), anti-GST (SC-138; Santa Cruz Biotechnology, USA), anti-Catalase (ab209211; Abcam, UK), anti-Actin (SC-47778; Santa Cruz Biotechnology), anti-Pex5 (GTX109798; GeneTex, USA), anti-Pmp70 (ab3421; Abcam), anti-HA (sc-7392; Santa Cruz Biotechnology), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, and Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (A11008 and A21422; Thermo Fisher Scientific, USA).

Identification of PS binding proteins

For screening novel PS binding proteins expressed in macrophages, Lysates of mouse BMDMs were used. The lysates were pre-incubated with 0.2 μM biotin-PC (L-11B16; Echelon Biosciences, USA) and 20 μl SCMBs (11205D; Invitrogen) at 4°C for 12 h to get rid of non-specific binding proteins. After that, the supernatants were incubated with 0.2 μM biotin-PS (L-31B16; Echelon Biosciences) and SCMBs 20 μl or SCMB 20 μl for 2 h. Bound proteins on SCMBs were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Specific bands shown in the biotin-PS sample were excised and analyzed through liquid chromatography-mass spectrometry (LC-MS).

Membrane lipid strip

Membrane lipid strips were purchased from Echelon Biosciences (P-6002) and the binding assay was performed according to manufacturer's protocol. To avoid non-specific binding, the membrane lipid strip was blocked for 1 h with

3% bovine serum albumin (BSA) in 10 ml TBS-T. Lysates of 293T cells overexpressing Hsd17b4 were incubated with the membrane lipid strip at 4°C for 12 h. After incubation, the strip was washed thoroughly, and bound Hsd17b4 to lipids on the strip was detected with an anti-Hsd17b4 antibody.

Immunoblotting and pull-down assay

Lysates of 293T cells overexpressing Hsd17b4 or BMDMs were incubated with 0.2 μM biotin-PS and 20 μl SCMBs at 4°C for 2 h. In order to test the effect of Ca^{2+} on Hsd17b4 binding to PS, 2.5 mM Ca^{2+} or 10 μM EGTA was additionally added to the lysis and wash buffer. To identify a part of PS binding to Hsd17b4, the lysates were incubated with 10 μM 12:0 N-Biotinyl fatty acid (860557P-5mg; Avanti Polar Lipids, USA) and SCMBs. Biotin-PS, SCMBs, and one of putative competitors for the association between Hsd17b4 and PS, 50 μM Glycerol 3-phosphate (94124; Sigma-Aldrich, USA), 1 mM Phospho-L-serine, 1 mM phosphor-D-serine, 2 μM DPPC (850355C; Avanti Polar Lipids), 2 μM DOPS (840035C; Avanti Polar Lipids), or liposomes, were incubated with the lysates. To test whether the topology of PS is crucial for the association of Hsd17b4 with PS, styrene beads coated with PS (P-B000 and P-B0PS; Echelon Biosciences) were also used and the binding assay was performed as described above. After incubation, beads were extensively washed and bead bound proteins were detected by immunoblotting.

PC, PS, and PC/PS (8:2) liposomes were prepared as previously described (Lee et al., 2019). Briefly, DPPC, or mixed DOPS with DPPC in chloroform (850355C and 840035C; Avanti Polar Lipids) was prepared. Chloroform was evaporated using Speed Vac (Thermo Fisher Scientific). Then, the pellets were reconstituted with 200 μl of phosphate-buffered saline (PBS) and sonicated.

PS exposure on BMDMs and Jurkat cells

BMDMs were suspended with Dulbecco's PBS (DPBS) without Ca^{2+} and Mg^{2+} and treated with 10 μM of A23187 (C7522; Sigma-Aldrich) at 37°C for 15 min. Jurkat cells were suspended in DPBS, irradiated with ultraviolet C (UVC), and incubated at 37°C for 2 h. BMDMs and jurkat cells were stained with AnnexinV-FITC (556419; BD, USA) and analyzed using flow cytometry (BD FACS Canto II).

Immunostaining

BMDMs were plated on the 18-mm diameter glass coverslips in a 12-well non-culture plate. The day after plating, the cells were washed with PBS, fix in 4% paraformaldehyde, and permeabilized with 0.1% TritonX-100. After 1 h blocking with 1% BSA, anti-Catalase or anti-Pmp70 and anti-Hsd17b4 antibodies were incubated with the cells at 4°C for 12 h. Then, the cells were washed twice with PBS and stained with alexa fluor 488 and 555-conjugated secondary antibodies. Images were acquired with Zeiss LSM 880 NLO (Zeiss, Germany).

Peroxisome enrichment

Peroxisomes were enriched in lysates of BMDMs treated with A23187 or apoptotic jurkat cells through serial centrifugation. Nuclei and debris were removed through 1,000*g* centrifugation. Then, heavy mitochondria were removed through 2,000*g* centrifugation. Peroxisomes in the remaining supernatants were further enriched by centrifugation at 25,000*g* and then, the pellets were considered as the fraction containing peroxisomes.

Efferocytosis assay

Efferocytosis assay was performed as previously described (Moon et al., 2020a). Briefly, LR73 cells were transfected with the indicated plasmids using lipofectamin 2000 (11668019; Invitrogen). One day after transfection, the cells were incubated with TAMRA-labeled (C1171; Life Technologies, USA) apoptotic thymocytes 2 h. Then, the cells were extensively washed with ic-cold PBS to remove unbound apoptotic cells, trypsinized, and analyzed with flow cytometry (BD FACS Canto II). For the double feeding assay, LR73 cells were incubated with unlabeled apoptotic thymocytes (the ratio of apoptotic cells to phagocytes is 1:1) for 24 h and washed with PBS to remove unbound apoptotic cells. Then the cells were further incubated with TAMRA-labeled apoptotic thymocytes (the ratio of apoptotic cells to phagocytes is 20:1) for 2 h, washed, trypsinized, and analyzed with flow cytometry.

RESULTS

Identification of Hsd17b4 as a PS-binding protein

To search for PS-binding proteins, lysates of BMDMs were pre-incubated with biotin-linked PC (biotin-PC) and SCMBs to remove non-specific binding proteins. Thereafter, the lysates were incubated with biotin-linked PS (biotin-PS) and SCMBs or SCMBs alone (Fig. 1A). Precipitated proteins were separated by SDS-PAGE and compared (Fig. 1B). A protein band specifically detected in the presence of biotin-PS was excised and analyzed by LC-MS. Hsd17b4 was the protein with the highest confidence (Supplementary Table S1). To test whether biotin-PS with SCMBs precipitated Hsd17b4, lysates of 293T cells overexpressing Hsd17b4 were incubated with SCMBs with or without biotin-PS. Hsd174b4 was detected in precipitants in the presence of biotin-PS, but not in the absence of biotin-PS (Fig. 1C). In addition, biotin failed to precipitate Hsd17b4 (Fig. 1D), indicating that Hsd17b4 is likely precipitated by PS on the beads. Precipitation of Hsd17b4 by PS was further confirmed with endogenous Hsd17b4. SCMBs with biotin-PS precipitated endogenous Hsd17b4, but SCMBs without biotin-PS did not (Fig. 1E). These data suggest that Hsd17b4 associates with PS.

Hsd17b4 binds to PS and cardiolipin

PS binds to various types of proteins including membrane and secreted proteins (Boada-Romero et al., 2020; Hanayama et al., 2002; Lemke, 2013). Most of these proteins require calcium for their binding to PS (Stace and Ktistakis, 2006). Thus, we tested whether calcium is also necessary for binding of Hsd17b4 to PS. Neither depletion of calcium using EGTA nor addition of calcium altered the binding of Hsd17b4 to PS, indicating that calcium is dispensable for this association (Figs. 2A and 2B). Next, we tested whether Hsd17b4 also binds to other lipids using a lipid membrane strip. The membrane strip was incubated with lysates of 293T cells overexpressing

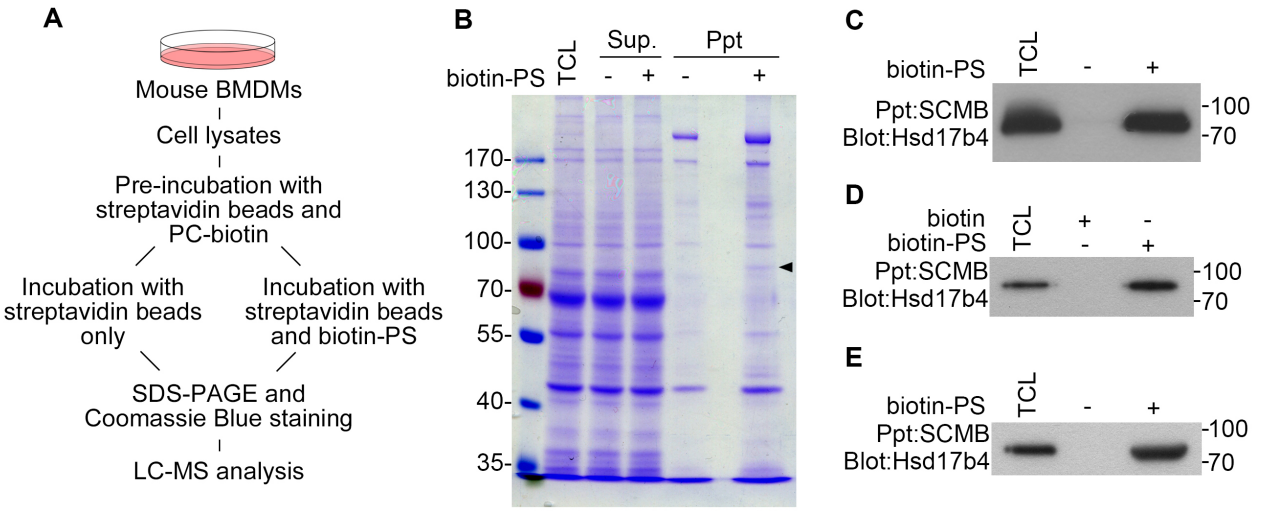

Fig. 1. Identification of Hsd17b4 as a PS-binding protein. (A) Schematic diagram of the assay used to identify PS-binding proteins. (B) Lysates of BMDMs were incubated with SCMBs in the presence (+) or absence (-) of biotin-linked PS for 2 h. Bound proteins were separated by SDS-PAGE. The arrowhead indicates a band specifically detected in precipitants in the presence of PS. TCL, total cell lysates; Sup., supernatants after precipitation; Ppt, precipitation. (C and D) Lysates of 293T cells overexpressing Hsd17b4 were incubated with SCMBs and biotin-PS (C) or biotin (D) for 2 h. Bound proteins were separated by SDS-PAGE and detected by immunoblotting with an anti-Hsd17b4 antibody. (E) Lysates of BMDMs were incubated with SCMBs and biotin-PS for 2 h. Precipitated proteins were detected by immunoblotting with an anti-Hsd17b4 antibody.

Hsd17b4, and binding of Hsd17b4 to a lipid(s) on the membrane strip was detected with an anti-Hsd17b4 antibody. Hsd17b4 bound strongly to PS and cardiolipin, and more weakly to phosphatidic acid (PA) and phosphatidylinositol derivatives, but not to other positively or negatively charged lipids on the membrane strip (Fig. 2C). These data suggest that Hsd17b4 preferentially binds to PS.

PS liposomes impair the association of Hsd17b4 with PS

We next investigated which region of Hsd17b4 is important for its association with PS. To this end, we generated Hsd17b4 truncation mutants containing the dehydratase, hydratase, or Scp-2-like domain of Hsd17b4 fused to the C-terminus of glutathione S-transferase (GST) (Fig. 2D), and tested whether PS precipitated the mutants. The Scp-2-like domain of Hsd17b4 was precipitated by PS, but the other truncation mutants were not (Fig. 2E), suggesting that the Scp-2-like domain is crucial for the association of Hsd17b4 with PS.

We next tested which part of PS is responsible for its association with Hsd17b4. PS consists of two fatty acid chains and phospho-serine attached to the glycerol backbone. In addition, Hsd17b4 is involved in beta-oxidation of long-chain fatty acids in peroxisomes (Pierce et al., 2010; Violante et al., 2019). Thus, we hypothesized that Hsd71b4 binds to the fatty acids of PS. To test this hypothesis, lysates of BMDMs were incubated with biotin-linked lauric acid and SCMBs. However, Hsd17b4 was not precipitated by this fatty acid but was robustly precipitated by PS (Fig. 3A), indicating that a fatty acid is not part of the region of PS to which Hsd17b4 binds. This is consistent with the finding that not all lipids containing fatty acids bound to Hsd17b4 (Fig. 2C). Next, we sought to identify which other regions of PS are required for its association with Hsd17b4. A region of PS required for its association with Hsd17b4 may disrupt this association due to competitive binding to Hsd17b4. Therefore, we evaluated whether the association between Hsd17b4 and PS was attenuated by the other parts of PS, namely, sn-glycerol 3-phosphate (sn-G3P) and phospho-serine, which are the glycerol backbone and head group of PS, respectively. The association between PS and Hsd17b4 was unaltered in the presence of sn-G3P (Fig. 3B). In addition, neither phospho-L-serine nor phosphor-D-serine, which were used to test stereospecific binding of PS to Hsd17b4, abolished the association (Fig. 3C). These data suggest that Hsd17b4 recognizes the whole PS molecule, not a part of it. We thus tested whether free PS blocks the association of PS with Hsd17b4. Unexpectedly, free PS also failed to impair the association of PS with Hsd17b4 (Fig. 3D).

The polar head group of PS on SCMBs faces outwards because biotin is linked to a fatty acid chain of PS, meaning PS on SCMBs mimics PS in the plasma membrane. Therefore, we hypothesized that the topology of PS in the plasma membrane is essential for the association of PS with Hsd17b4. To test this hypothesis, we used liposomes in which the topology of PS was similar to that of PS in the plasma membrane and on SCMBs. PS and PS/PC liposomes drastically attenuated the association of PS with Hsd17b4, whereas PC liposomes did not (Fig. 3E). To confirm that the topology of PS in the plasma membrane is crucial for the association of PS with Hsd17b4, we used PS-coated styrene beads on which PS was randomly oriented. PS-coated styrene beads precipitated substantially less Hsd17b4 than SCMBs, on which PS was directionally immobilized (Fig. 3F). Taken together, these data

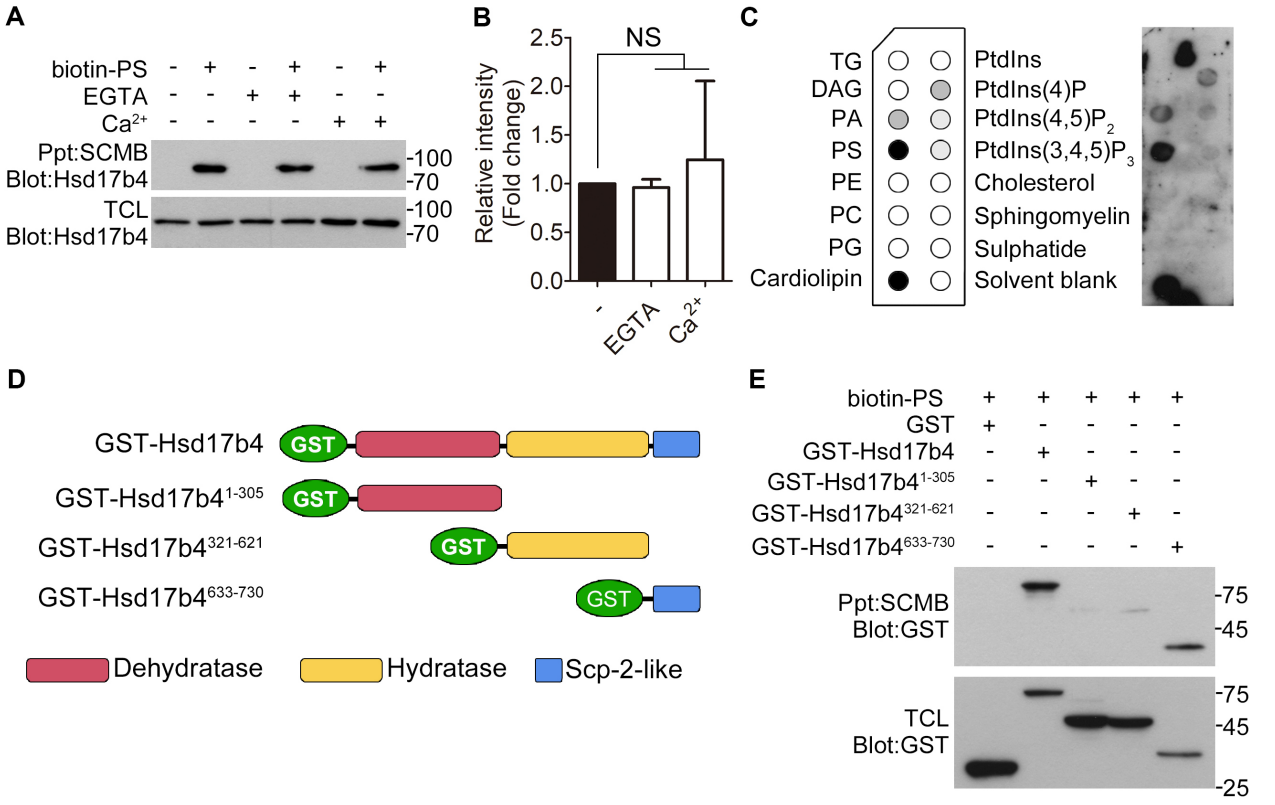

Fig. 2. The Scp-2-like domain of Hsd17b4 is crucial for binding to PS. (A and B) Lysates of BMDMs were incubated with SCMBs and biotin-PS in the presence of EGTA (10 μM) or calcium (2.5 mM) for 2 h. Precipitated Hsd17b4 was detected by immunoblotting with an anti-Hsd17b4 antibody (A) and was quantified (B). TCL, total cell lysates; NS, not significant. (C) Lipids spotted on a nitrocellulose membrane strip were incubated with lysates of 293T cells overexpressing Hsd17b4 for 2 h. Binding of Hsd17b4 to PS and other lipids was detected by immunoblotting with an anti-Hsd17b4 antibody. TG, triacylglycerol; DAG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol. (D) Schematic diagram of the truncation mutants of Hsd17b4. (E) 293T cells transfected with the indicated plasmids were lysed. Lysates were incubated with SCMBs and biotin-PS. Bound proteins were detected by immunoblotting with an anti-GST antibody.

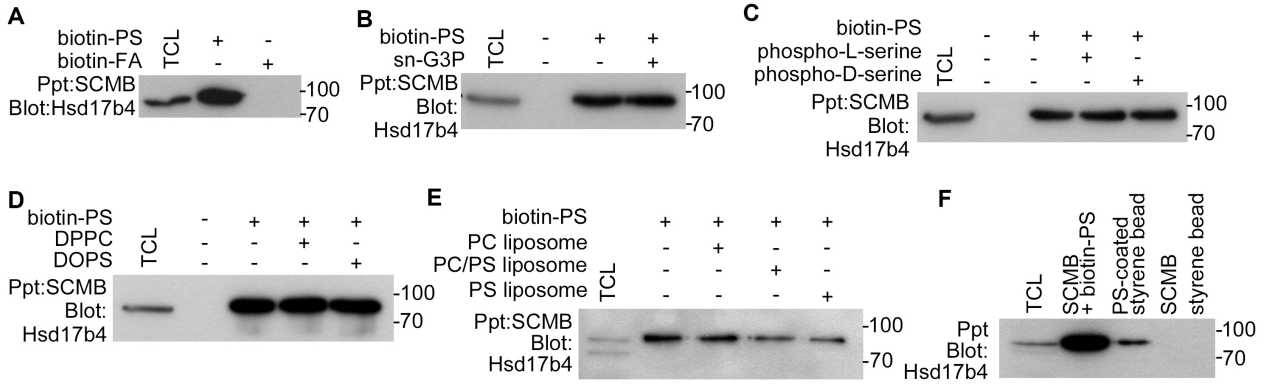

Fig. 3. PS liposomes impair the association between PS and Hsd17b4. (A) Lysates of BMDMs were incubated with SCMBs and biotin-PS or biotin-lauric acid. Bound proteins were detected by immunoblotting with an anti-Hsd17b4 antibody. TCL, total cell lysates. (B-D) Lysates of BMDMs were incubated with SCMBs in the presence of sn-G3P (B), the indicated phospho-serine (C), or the indicated lipids (D). Precipitated Hsd17b4 was detected by immunoblotting. sn-G3P, sn-glycerol 3-phosphate; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine. (E) Lysates of BMDMs were incubated with SCMBs and biotin-PS in the presence of the indicated liposomes. Bound proteins were detected by immunoblotting with an anti-Hsd17b4 antibody. (F) Lysates of BMDMs were incubated with SCMBs and biotin-PS or with PS-coated styrene beads. Bound proteins were detected by immunoblotting with an anti-Hsd17b4 antibody.

imply that Hsd17b4 perceives the topology of PS in the membrane, which is essential for the association of Hsd17b4 with PS.

PS regulates the localization of Hsd17b4 to peroxisomes

PS is exclusively located in the inner leaflet of the plasma membrane but translocates to the outer leaflet under certain conditions, e.g., apoptosis (Fadok et al., 1992). Thus, it is possible that the location of PS in the inner or outer leaflet of the plasma membrane affects the subcellular localization of Hsd17b4. To test this possibility, we used A23187, a calcium ionophore, to induce translocation of PS from the inner to the outer leaflet of the plasma membrane. Treatment of cells with A23187 induced exposure of PS on the cell surface (Fig. 4A). We investigated whether the subcellular location of Hsd17b4 is altered in cells treated with A23187. Hsd17b4 and Catalase, a peroxisomal marker, localized to puncta but their fluorescent signals were not superimposed in control cells. However, fluorescent signals of Hsd17b4 colocalized more with fluorescent signals of Catalase in A23187-treated cells than in control cells (Fig. 4B). In addition, the level of Hsd17b4 was higher in the peroxisomal fraction of A23187-treated cells than in the peroxisomal fraction of control cells (Fig. 4C). When Pmp70, another peroxisomal maker, was used instead of catalase, Hsd17b4 colocalized with Pmp70 more than Catalase at the basal level. Nevertheless, treatment of cells with A23187 robustly increased colocaliztion of Hsd17b4 with Pmp70 (Supplementary Fig. S1), suggesting that translocation of PS induces the peroxisomal localization of Hsd17b4.

Next, we investigated whether the subcellular location of Hsd17b4 is altered during apoptosis. Cells treated with ultraviolet (UV) irradiation expose PS on their surface and undergo apoptosis. Jurkat cells irradiated with UV exhibited exposure of PS (Fig. 4D), and the level of Hsd17b4 was higher in the peroxisomal fraction of apoptotic Jurkat cells than in the peroxisomal fraction of live Jurkat cells (Fig. 4E). Pex5, also called peroxisomal targeting sequence 1 receptor, binds to the C-terminal peroxisomal targeting sequence of proteins imported to peroxisomes and plays an essential role in per-

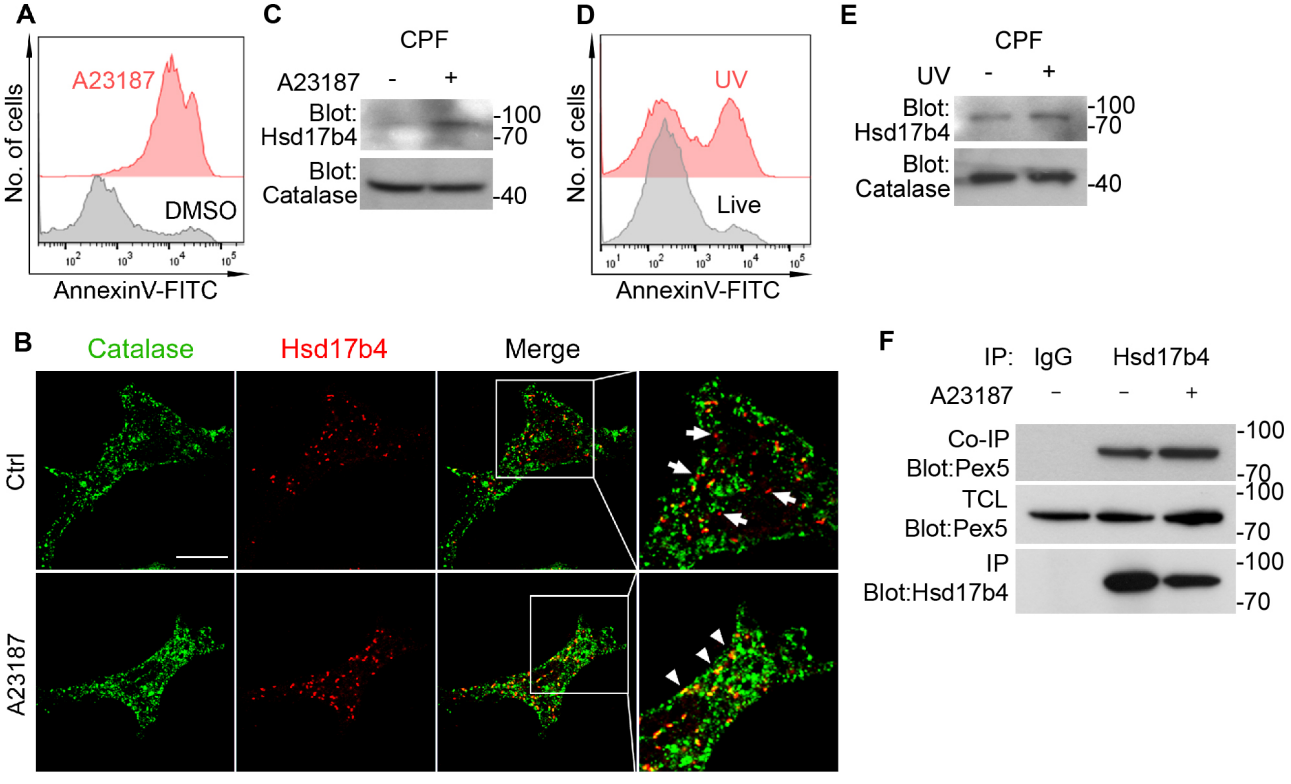

Fig. 4. Translocation of PS enriches Hsd17b4 in peroxisomes. (A) BMDMs treated with A23187 (10 μM) were stained with FITC-labeled Annexin V and analyzed by flow cytometry. (B) BMDMs treated with A23187 (10 μM) were incubated with anti-Hsd17b4 and anti-Catalase antibodies and then stained with Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies. Images were acquired by confocal microscopy. Arrowheads indicate puncta where Hsd17b4 colocalizes with Catalase and arrows indicate puncta where Hsd17b4 does not colocalize with Catalase. Ctrl, control. Scale bar = 20 μm. (C) Lysates of BMDMs treated with A23187 (10 μM) were fractionated. Proteins in the fraction containing peroxisomes were detected by immunoblotting with the indicated antibodies. CPF, crude peroxisomal fraction. (D) Jurkat cells were irradiated with UV, stained with Annexin-FITC, and analyzed by flow cytometry. (E) Jurkat cells irradiated with UV were lysed and fractionated. Proteins in the fraction containing peroxisomes were detected by immunoblotting with the indicated antibodies. (F) BMDMs treated with A23187 were lysed, and the lysates were incubated with protein A/G conjugated-agarose beads and an anti-Hsd17b4 antibody or an isotype control antibody. Beads bound proteins were detected using immunoblotting.

oxisomal protein import (Otera et al., 2002). Thus, we tested whether treatment of cells with A23187 alters interaction of Hsd17b4 with Pex5. Intriguingly, the level of coimmunoprecipiated Pex4 with Hsd17b4 in cells treated with A23187 was higher than that in control cells (Fig. 4F), indicating that translocation of PS promotes interaction of Hsd17b4 with Pex4. Collectively, these data suggest that the subcellular localization of Hsd17b4 is regulated by PS, which associates with and dissociates from Hsd17b4.

DISCUSSION

A key step during phagocytosis of apoptotic cells (efferocytosis) is recognition of apoptotic cells by phagocytes, which is mediated by an interaction between ligands on apoptotic cells and receptors on phagocytes (Boada-Romero et al., 2020; Lee et al., 2019). PS is the best-known ligand on apoptotic cells and is recognized by PS receptors on phagocytes or by binding of bridging molecules to PS on apoptotic cells and receptors on phagocytes. Due to the importance of PS and its receptors during efferocytosis, studies have sought to identify PS receptors over the past decades (Boada-Romero et al., 2020). Initially, we also attempted to identify a PS-binding protein expressed on the plasma membrane. Unexpectedly, however, we identified Hsd17b4, a peroxisomal protein, as a PS-binding protein. At the beginning of this study, we tested the role of Hsd17b4 in efferocytosis due to the metabolic link between Hsd17b4 and efferocytosis (Park et al., 2011; Wang et al., 2017; Yurdagul et al., 2020). Specifically, engulfed apoptotic cells are degraded and metabolized in phagocytes, and Hsd17b4 is involved in peroxisomal beta-oxidation (Pierce et al., 2010; Violante et al., 2019). To test the effect of Hsd17b4 on efferocytosis, Hsd17b4 was expressed in LR73 cells, and efferocytosis was evaluated. Phagocytosis of apoptotic cells by Hsd17b4-overexpressing LR73 cells was similar to that by control cells, as measured by the percentage and mean fluorescence intensity (MFI; proportional to the number of engulfed apoptotic cells per phagocyte) of phagocytes engulfing apoptotic cells (Figs. 5A and 5B). Continuous engulfment of apoptotic cells by phagocytes is enhanced by decreasing the metabolic load (Park et al., 2011; Wang et al., 2017). We also tested whether Hsd17b4 promotes continuous efferocytosis. However, Hsd17b4 overexpression did not affect efferocytosis by LR73 cells loaded with apoptotic cells (Figs. 5C-5E), suggesting that neither Hsd17b4 nor peroxisomal beta-oxidation mediated by Hsd17b4 is involved in efferocytosis.

At the beginning of the study, we tried to observe the colocalization of Hsd17b4 with PS in cells using immunocytochemistry, but failed. The failure may be caused by competition for binding to PS between Hsd17b4 and PS binding proteins used for PS labeling. Proteins including an anti-PS antibody that specifically bind to PS recognize the head group of PS, phospho-L-serine, to which Hsd17b4 may also bind due to its specificity to PS. Thus, it is not feasible to label PS associating with Hsd17b4. Indeed, we observed that the IgV domain of Tim-4, which is known to bind to PS, attenuated the interaction of Hsd17b4 with PS (Supplementary Fig. S2). It seems that methodological advance is required to visualize the colocalization of Hsd17b4 with PS.

In this study, we found that translocation of PS from the inner to the outer leaflet of the plasma membrane altered the subcellular localization of Hsd17b4. However, we did not address the consequence of Hsd17b4 localization to peroxisomes. It was recently reported that ceramide binds to Hsd17b4. Depletion of ceramide results in translocation of Hsd17b4 to peroxisomes, and this is related to enhanced production of docosahexaenoic acid (DHA) and eicosapentae-

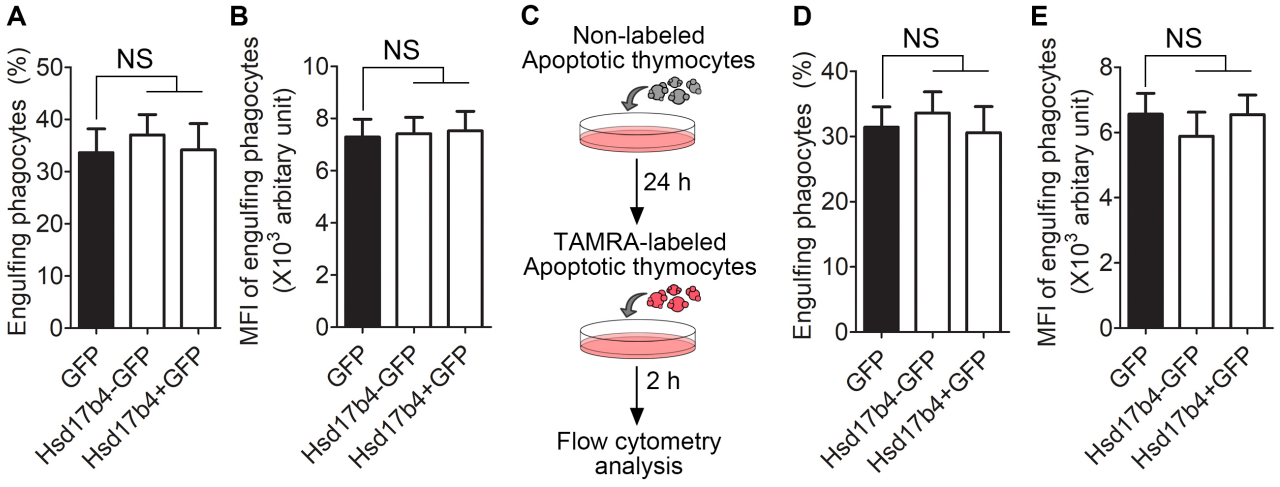

Fig. 5. Hsd17b4 is unlikely to be involved in efferocytosis. (A and B) LR73 cells transfected with the indicated plasmids were incubated with TAMRA-labeled apoptotic thymocytes for 2 h, extensively washed with ice-cold PBS, trypsinized, and analyzed by flow cytometry. Cells double-positive for GFP and TAMRA were considered to be phagocytes engulfing apoptotic cells. NS, not significant. (C) Schematic diagram of the assay used to assess continuous efferocytosis. (D and E) LR73 cells transfected with the indicated plasmids were incubated with the same number of unstained apoptotic cells. After incubation for 24 h, LR73 cells were incubated with TAMRA-stained apoptotic cells for 2 h, and then LR73 cells engulfing TAMRA-stained apoptotic cells were analyzed by flow cytometry.

noic acid (EPA), which are generated in peroxisomes. Thus, Hsd17b4 is critical for peroxisomal generation of DHA and EPA (Zhu et al., 2019). In addition, it is known that Hsd17b4 also binds to Vitamin K2 and Chromeceptin, a synthetic molecule impairing the viability and growth of IGF2-overexpressing hepatocellular carcinoma cells and is involved in estrogen metabolism and Chromeceptin-mediated signaling in IGF2-overexpressing hepatocellular cells (Choi et al., 2006; Otsuka et al., 2005). Localization of Hsd17b4 to peroxisomes triggered by translocation of PS during apoptosis might elicit analogous effects on DHA, EPA, and estrogen metabolism and Chromeceptin-mediated signaling. PS is exposed not only on dying cells, but also on living cells. For example, PS translocates from the inner to the outer leaflet of the plasma membrane in activated platelets, sperm, aged red blood cells, activated lymphocytes, and some cancer cells (Elliott et al., 2005; Lentz, 2003; Qadri et al., 2017; Riedl et al., 2011; Rival et al., 2019). Thus, PS could be translocated to the outer leaflet of the plasma membrane in these cells, which alters subcellular localization of Hsd17b4 and turns Hsd17b4-mediated signaling on and/or off. It would be interesting to investigate whether the subcellular localization of Hsd17b4 is changed in these cells and, if so, how alteration of Hsd17b4 localization affects their functions.

In the study, we found that biotin-linked PS on SCMBs precipitated more Hsd17b4 than PS on styrene beads, and only PS liposomes impaired the association of PS with Hsd17b4. These findings imply that the topology of PS in the membrane is essential for its association with Hsd17b4. However, we also observed binding of Hsd17b4 to PS spotted on a nitrocellulose membrane. This result might be due to the hydrophilic property of the nitrocellulose membrane. The hydrophilicity of the membrane might generate a layer of phospholipids that form the appropriate topology of PS required for the association of PS and Hsd17b4. Taken together, this study demonstrates that Hsd17b4 is a novel PS-binding protein and suggests that PS alters the subcellular location of Hsd17b4.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This research was funded by the National Research Foundation of Korea funded by the Korea government (MSIP) (2019R1A2C1006480, 2019R1I1A1A01057419, and 2019R1A4A1028802) and by GIST Research Institute (GRI) ARI grant in 2020.

AUTHOR CONTRIBUTIONS

S.A.L. and D.P. conceived this study and wrote the manuscript. S.A.L., J.L., K.K., H.M., C.M., B.M., D.K., S.Y., and H.P. performed experiments and analyzed data. J.L. and D.P. acquired funding. R.P. and G.L. provided reagents and expertise.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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